Attorney Docket No. 21486-032001US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT :	Wands et al.		
SERIAL NUMBER :	09/436,184	EXAMINER:	Karen Canella
FILING DATE :	November 8, 1999	ART UNIT :	1642
FOR :	DIAGNOSIS AND TREATMENT OF MALIGNANT NEOPLASMS		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF JACK R. WANDS AND SUZANNE DE LA MONTE UNDER 37 C.F.R §1.132

We, Jack R. Wands and Suzanne de la Monte, of East Greenwich, Rhode Island, declare and state as follows:

1. We are co-inventors of the invention claimed in the above-referenced application and are employed by the named assignee, Rhode Island Hospital, Providence, Rhode Island.

2. I, Jack R. Wands, received a M.D. degree from the University of Washington in 1969 and currently serve as Chief of the Division of Gastroenterology at Lifespan Rhode Island Academic Medical Center, Director of the Liver Research Center, Professor of Medicine at Brown University School of Medicine, and Head of the Gastroenterology Section at Brown University. I am a member of the editorial boards of the academic journals Hepatology, International Hepatology Communications, Journal of Viral Hepatitis, and Viral Hepatitis Reviews, and serve as an editorial consultant for the Journal of Clinical Investigation, New England Journal of Medicine, Proceedings of the National Academy of Sciences, Journal of Infectious Disease, Gastroenterology, Journal of Virology, Virology and Nature Medicine. I have been involved in research relating to cancer for over 30 years.

3. I, Suzanne de la Monte, received my undergraduate degree from Cornell University, M.D. from Cornell University Medical College, and M.PH. from The Johns Hopkins School of Hygiene and Public Health. I completed my Anatomic Pathology residency at Johns Hopkins Hospital and Neuropathology fellowship at the Massachusetts General Hospital/ Harvard Medical School. I have been actively involved in directing research in the field of molecular mechanisms of cell migration/invasion for at least 15 years, and serve on the editorial boards of several biomedical journals.

4. We have read the Office Action mailed on January 15, 2008 and are familiar with the Examiner's grounds of rejection of the pending claims.

5. Overexpression of aspartyl (asparaginyl) beta-hydroxylase (AAH) is involved in tumor growth and invasiveness. Inhibition of AAH expression led to reduced tumor growth in an animal model.

6. 9L Gliosarcoma cells were transfected in vitro with AAH antisense oligonucleotide and empty vector using the streptolysin O (SLO) method. The cells were incubated overnight. Six rats were inoculated with 9L cells containing the AAH oligonucleotide. Six rats received 9L cells containing empty vector.

7. The rats were sacrificed daily from day 4 to day 10 of tumor inoculation. Brains were harvested. A section through the site of inoculation was snap frozen and also saved in histofix for histologic preparations.

8. Results of Mock (negative control) Inoculation demonstrated that glioblastoma cells proliferated rapidly creating a large mass in the brain. Moreover, glioblastoma cells invaded brain tissue with finger-like projections. Rats also became moribund within 18 days after tumor cell inoculation, as expected.

9. Results of AAH antisense therapy demonstrated that after 7 days, tumor mass was substantially smaller (50-75%) than in the control brains. Moreover, brains inoculated with

AAH- oligonucleotide treated cells showed low tumor cell density compared with control tumor cell density. Tumors comprised of AAH treated glioblastoma cells did not form finger-like projections.

10. In conclusion, the antisense AAH therapy led to reduced glioblastoma cell to proliferation *in vivo*. As a result of antisense AAH therapy, tumors masses were substantially reduced in size relative to control tumor masses, and in 20% of the animals, the tumors failed to grow. The small tumor masses that did grow during antisense AAH therapy lacked the finger-like projections seen in the control tumors, which are indicative of tumor invasion. In contrast to the control population, rats that were treated using AAH antisense therapy were not moribund 17 days post tumor cell inoculation. These data indicate that the claimed methods inhibit tumor growth, progression, and invasiveness.

11. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by a fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date July 15, 2008 Jack R. Wands

Date 15 July 2008 Suzanne de la Monte

The AAPS Journal 2005; 7 (1) Article 9 (http://www.aapsj.org).

DNA-based Therapeutics and DNA Delivery Systems: A Comprehensive Review

Submitted: February 6, 2004; Accepted: April 8, 2004; Published: April 8, 2005.

Siddhesh D. Patil,[1] David G. Rhodes,[1] and Diane J. Burgess[1]

[1]Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269.

ABSTRACT

The past several years have witnessed the evolution of gene medicine from an experimental technology into a viable strategy for developing therapeutics for a wide range of human disorders. Numerous prototype DNA-based biopharmaceuticals can now control disease progression by induction and/or inhibition of genes. These potent therapeutics include plasmids containing transgenes, oligonucleotides, aptamers, ribozymes, DNAzymes, and small interfering RNAs. Although only 2 DNA-based pharmaceuticals (an antisense oligonucleotide formulation, Vitravene, (USA, 1998), and an adenoviral gene therapy treatment, Gendicine (China, 2003), have received approval from regulatory agencies; numerous candidates are in advanced stages of human clinical trials. Selection of drugs on the basis of DNA sequence and structure has a reduced potential for toxicity, should result in fewer side effects, and therefore should eventually yield safer drugs than those currently available. These predictions are based on the high selectivity and specificity of such molecules for recognition of their molecular targets. However, poor cellular uptake and rapid in vivo degradation of DNA-based therapeutics necessitate the use of delivery systems to facilitate cellular internalization and preserve their activity. This review discusses the basis of structural design, mode of action, and applications of DNA-based therapeutics. The mechanisms of cellular uptake and intracellular trafficking of DNA-based therapeutics are examined, and the constraints these transport processes impose on the choice of delivery systems are summarized. Finally, the development of some of the most promising currently available DNA delivery platforms is discussed, and the merits and drawbacks of each approach are evaluated.

KEYWORDS: nucleic acid therapeutics, DNA delivery systems, nonviral vectors, viral vectors, liposomes, gene therapy.

INTRODUCTION

The DNA molecule has been one of the most important sources not only for the understanding of the fundamental basis of human life but also for the development of a novel group of therapeutics modeled on its endogenous structure. DNA-based therapeutics include plasmids containing transgenes for gene therapy, oligonucleotides for antisense and antigene applications,[1] ribozymes, DNAzymes, aptamers, and small interfering RNAs (siRNAs).[2,3] Although most of the DNA-based drugs are in early stages of clinical trials, this class of compounds has emerged in recent years to yield extremely promising candidates for drug therapy for a wide range of diseases, including cancer, AIDS, neurological disorders such as Parkinson's disease and Alzheimer's disease, and cardiovascular disorders.[2,3]

Elucidation of the human genome has also provided a major impetus in identifying human genes implicated in diseases, which may eventually lead to the development of DNA-based drugs for gene replacement or potential targets for gene ablation.[4] In addition, using genomic data, potent DNA-based drugs may be developed for individualized medicine.[5] The Human Genome Project will help determine genetic markers responsible for patient response to drug therapy, drug interactions, and potential side effects.[5] Developments in human genomics, transcriptomics, and proteomics will provide an additional impetus for the advancement of DNA-based therapeutics by supplying novel targets for drug design, screening, and selection.

One of the significant advantages of DNA-based drugs over currently available low molecular weight pharmaceuticals is their selective recognition of molecular targets and pathways, which imparts tremendous specificity of action. These drugs can be used to mitigate disease states either prophylactically or at a very early stage, thereby preventing disease progression and its complications. For example, gene therapy typically involves correction of a malfunctioning gene by the introduction and expression of its correct copy, thus resulting in a single protein product. Similarly, for DNA-based therapeutics intended for gene ablation, only selected genes are switched off, thus ensuring specificity in controlling the disease status. Therefore, at least theoretically, the potential for negative side effects of DNA-based therapeutics may be minimized compared to conventional pharmaceuticals that typically have less specificity. However, since many DNA-based compounds are new pharmaceutical drug candidates and the effects of their human exposure have yet to be completely investigated, the feasibility of their long-term use remains to be determined. In addition, very little is known about their cellular uptake, distribution, and metabolism.

Corresponding Author: Diane J. Burgess, Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269. Tel: 860-486-3760. Fax: 860-486-4998. Email: Diane.burgess@uconn.edu.

Table 1. DNA-based Therapeutics Currently in Advanced Stages of Clinical Development (Phase 3 and Beyond)*

Drug Candidate - Company	Type of DNA-based Therapeutic	Development Status	Molecular Basis of Action	Disease Indication
Gendicine - SiBiono Genetech	Plasmid	Chinese-FDA approved	Adenovirus encoding the tumor suppressor p53 gene	Head and neck squamous cell carcinoma
Advexin - Introgen Therapeutics	Plasmid	Phase 3 alone or in combination with cisplatin and 5-fluorouracil	Adenovirus encoding the tumor suppressor p53 gene	Refractory head and neck squamous cell carcinoma
Vitravene (fomivirsen sodium) - Isis Pharmaceuticals	Antisense oligonucleotide	FDA approved	Inhibitor of immediate early region 2 (IE2) of human cytomegalovirus	Cytomegalovirus retinitis in AIDS patients
Affinitak - Isis Pharmaceuticals	Antisense oligonucleotide	Phase 3 in combination with carboplatin and paclitaxel	Inhibitor of protein kinase c-alpha (PKC-alpha) expression	Stage IIIb or Stage IV non-small cell lung cancer
Alicaforsen - Isis Pharmaceuticals	Antisense oligonucleotide	Phase 3	Inhibitor of Intracellular Adhesion Molecule -1 (ICAM-1)	Crohn's disease
Macugen (pegaptanib sodium) - Eyetech and Pfizer	Aptamer	Phase 3 with or without photodynamic therapy	Inhibitor of vascular endothelial growth factor (VEGF)	Age-related macular degeneration
Genasense (oblimersen sodium) - Aventis and Genta	Antisense oligonucleotide	Late Stage phase 3 in combination with dexamethasone	Inhibitor of B cell leukemia/lymphoma 2 (Bcl-2) protein	Malignant melanoma
Genasense (oblimersen sodium) - Aventis and Genta	Antisense oligonucleotide	Phase 3 in combination with fludarabine and cyclophosphamide	Inhibitor of B cell leukemia/lymphoma 2 (Bcl-2) protein	Chronic lymphocytic leukemia
Genasense (oblimersen sodium) - Aventis and Genta	Antisense oligonucleotide	Phase 3 in combination with dacarbazine	Inhibitor of B cell leukemia/lymphoma 2 (Bcl-2) protein	Multiple myeloma

*All development statuses are filed with the United States Food and Drug Administration (USFDA) unless otherwise indicated.

Despite many favorable characteristics and signs of possible clinical victories (see Table 1), the introduction of DNA-based drugs for human use can be best described as limited, with rare successes.[3] The inertia in the development of these drugs can be attributed, in part, to their poor cellular uptake profile in vivo. The innate ability of DNA-based drugs to be internalized by target cells is minimal under normal circumstances. In addition, poor biological stability and a short half-life result in unpredictable pharmacokinetics. Furthermore, DNA molecules that do manage to enter the cell are subsequently subjected to intracellular degradation along with stringently restricted nuclear access. The resulting random delivery profile of DNA-based drugs is further complicated by a lack of in vivo/in vitro correlation of their pharmacological outcomes.

Over the past several years, understanding of DNA uptake, trafficking, and metabolism has been considerably enhanced, and several strategies have been deployed to improve the stability and minimize the degradation of DNA-based therapeutics. Chemical derivatization has yielded excellent results for improving the biological stability of short-length DNA-based therapeutics such as oligonucleotides. High molecular weight compounds, such as plasmid DNA, have been successfully delivered into cells using both synthetic and natural delivery platforms. The use of DNA delivery systems has not only improved the pharmacokinetics of DNA-based therapeutics but has also achieved efficient targeted introduction of these molecules into desired tissues. Over the past several decades, the pursuit of the ideal DNA delivery system has yielded very promising results and a myriad of specialized delivery options. In this review, we summarize the design, mechanism of action, and some of the latest clinical applications of DNA-based therapeutics. The classes of DNA-based therapeutics and their cellular uptake and intracellular fate are discussed. In addition, some of the most promising DNA delivery systems are evaluated.

DNA-BASED THERAPEUTICS

Plasmids

Molecular Basis of Action

Plasmids are high molecular weight, double-stranded DNA constructs containing transgenes, which encode specific proteins.[6] On a molecular level, plasmid DNA molecules can be

considered pro-drugs that upon cellular internalization employ the DNA transcription and translation apparatus in the cell to biosynthesize the therapeutic entity, the protein.[6] Gene therapy involves the use of plasmid DNA to introduce transgenes into cells that inherently lack the ability to produce the protein that the transgene is programmed to generate. Plasmids can be used to correct genetic errors that produce functionally incompetent copies of a given protein (see Figure 1). The mechanism of action of plasmid DNA requires that the plasmid molecules gain access into the nucleus after entering the cytoplasm. The entry of plasmid molecules into the nucleus through the nuclear pores is an extremely challenging and difficult process.[6] Nuclear access or lack thereof eventually controls the efficiency of gene expression (see Figure 1).[6] In addition to disease treatment, plasmids can be used as DNA vaccines for genetic immunization.[7] DNA vaccines function through induction of immune response by introducing genes encoding antigens for specific pathogens.

Suicide gene therapy is another rapidly emerging strategy for induction of transgenes.[8-10] Briefly, this methodology involves the stable transfection of chemosensitization genes in tumor cells, which upon gene expression convert a separately administered, nontoxic pro-drug into a chemotoxic drug. Since only the transfected tumor cells are capable of this intracellular conversion, only they are susceptible to the chemotoxic entity, hence the term suicide gene therapy. For example, cytosine deaminase-based suicide gene therapy was investigated in a syngeneic rat model of peritoneal carcinomatosis.[11] It was demonstrated that when rats with tumor cells expressing the cytosine deaminase gene were administered the pro-drug 5-fluorocytosine, they converted it to cytotoxic 5-fluorouracil, a chemotherapeutic agent that led to remission of tumors and prolonged their life.[11] Suicide gene therapy has also been employed for gene-directed enzyme-pro-drug activation using such genes as herpes simplex type-1 thymidine kinase enzyme for antiviral pro-drugs gancyclovir and zidovudine and purine nucleoside phosphorylase for fludarabine.[8-10]

Plasmid Design

The design and engineering of plasmids to obtain maximum transfection has been extensively researched.[12] In addition to the transgene of interest, plasmid DNA molecules typically contain several regulatory signals such as promoter and enhancer sequences that play an important role in regulating gene expression.[13] In viral delivery vectors, such signals can be endogenously present or artificially engineered in the virus genome.[12] In addition, splicing and polyadenylation sites are present in the transgene construct that help in the correct processing of the mRNA generated after transcription.[12] Some vectors also have introns that may increase pre-mRNA processing and nuclear transport.[13]

Promoter sequences play a vital role in initiating gene transcription. Promoter sequences offer recognition sites for the RNA polymerase to initiate the transcription process. Higher efficiency can be obtained by engineering the plasmid with strong tissue- or tumor-specific promoters.[14] Commonly used promoter sequences are derived from viral origins such as cytomegalovirus (CMV) and roux sarcoma virus, or are obtained from human origins such as alpha actin promoter.[14] However, sometimes promoters can lose their activity upon immune stimulation.[15,16] Human promoters have been shown to have greater resiliency against immune response activation than viral promoters.[15] Promoter sequences may also play an important role in determining the immune response of the cell to the gene product.[17] For example, it was demonstrated that human muscle creatine kinase promoter has no immunostimulatory effect in mice, as opposed to the commonly used CMV promoter sequence, during the expression of a gene vaccine encoding the hepatitis B surface antigen.[17] Tissue-specific promoters can also improve the efficacy of suicide gene therapy.[18]

Enhancers are regions in the plasmid DNA that enhance the production of the gene of interest by as much as several hundred times.[14] Enhancers can be tissue specific and can be present on the plasmid locus either upstream or downstream from the promoter region. Transcription efficiency can be substantially improved by the choice of suitable enhancers. For example, promoters and enhancers derived from immunoglobulin genes have been used to increase gene transduction in hematopoietic cells and to improve specificity of viral vectors useful in the treatment of hematological malignancies.[14] Muscle creatine kinase enhancer has been useful for enhanced targeted expression of transgenes for gene therapy to correct for muscular dystrophy.[14]

Clinical Applications of Plasmids for Gene Therapy

The success of efficient gene transfer into mammalian cells in vitro and animal models in vivo encouraged early human gene therapy experimentation. In its early stages of development, plasmid-based gene therapy was attempted to correct inheritable disorders resulting from a single gene defect. Such disease targets included adenosine deaminase deficiency in severe combined immunodeficiency disease (SCID), cystic fibrosis transmembrane conductance regulator gene mutation in cystic fibrosis, hypoxanthine-guanine phosphoribosyl transferase deficiency in Lesch-Nyhan disease, and glucocerebrosidase enzyme deficiency in Gaucher disease.[19]

The first federally approved human gene therapy protocol was initiated in 1990 for the treatment of adenosine deaminase deficiency.[19] Since then, more than 500 gene therapy protocols have been approved or implemented.[20] As recently as 2002, scientists reported the successful gene-therapy-based cure for SCID.[21] In 2003, the Chinese drug regulatory agency

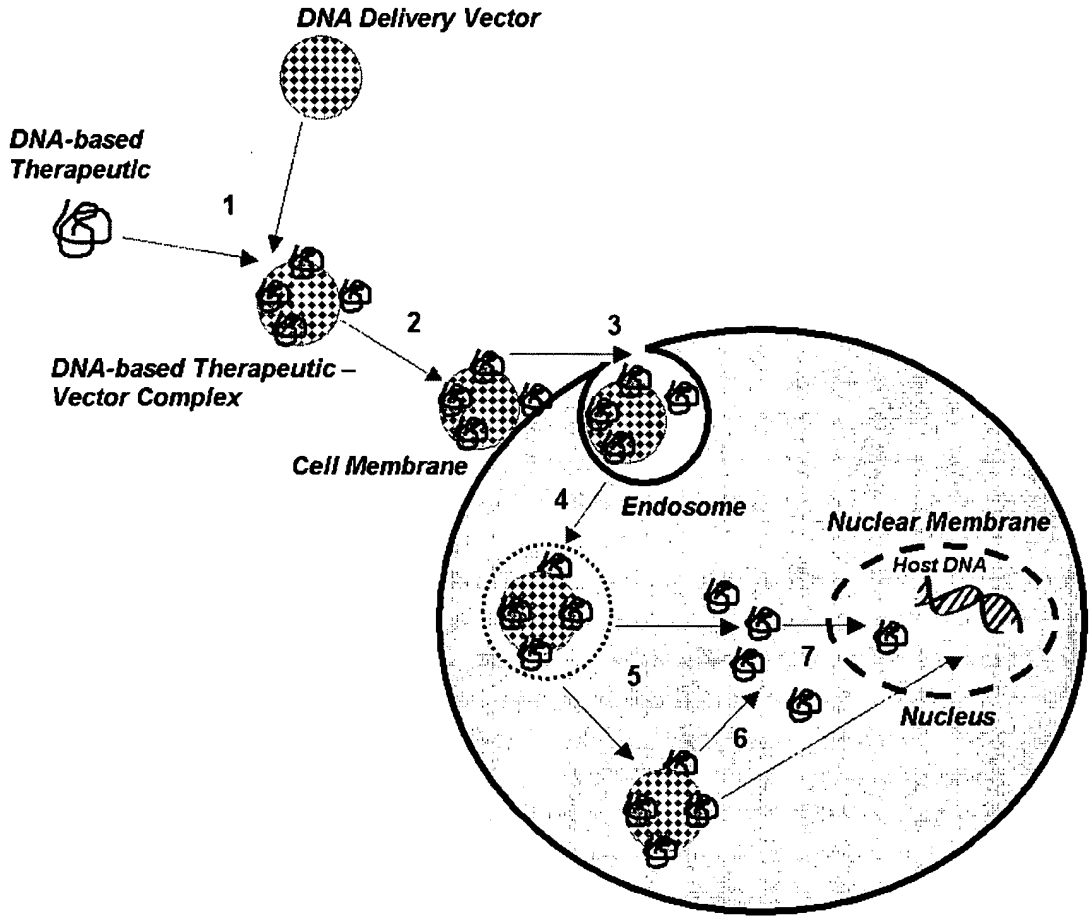

Figure 1. Schematic generalized representation of delivery of a DNA-based therapeutic using a viral or nonviral DNA delivery vector: (1) complexation and/or entrapment of DNA-based therapeutic with DNA delivery vector; (2) interaction of DNA-based therapeutic-vector complex with cell membrane; (3) cellular internalization via receptor- or non-receptor-mediated endocytotic pathways; (4) endosomal breakdown; (5) cytoplasmic release of DNA-based therapeutic-vector complex or DNA-based therapeutic alone (Cytoplasm is the site of action for antisense oligonucleotides, aptamer, ribozymes, DNAzymes, and cytoplasmic plasmid DNA expression systems); (6) dissociation of DNA-based therapeutic from vector; (7) nuclear translocation of viral vectors or DNA-based therapeutics. (Nucleus is the site of action for transgenes in plasmids for gene therapy, siRNA generating plasmids, and antigene oligonucleotides.)

Macromedia Flash animations of the following processes are available in the online version of this article: (A) Activity of an antigene oligonucleotide; (B) Activity of an antisense oligonucleotide; (C) Activity of an aptamer; (D) Activity of DNAzyme; (E) Activity of RNAzyme; (F) Activity of a siRNA; and (G) Activity of a plasmid therapeutic. The reader may access the interactive and animated user-controlled interface online, select the type of DNA-based therapeutic, and observe a detailed graphic animation of the specific processes involved in its uptake, subsequent intracellular fate, and mechanism of action.

To access the online version of this article go to http://www.aapsj.org/.

approved the first gene therapy product for head and neck squamous cell carcinoma, which will be marketed under the trade name Gendicine and will be available in early 2004[22] (see Table 1). Gendicine consists of an adenoviral gene delivery system that is capable of inserting the p53 gene into tumor cells, thereby stimulating cell death. Clinical trials using Gendicine demonstrated complete regression of tumors in approximately two thirds of volunteers with late-stage head and neck squamous cell carcinoma. This milestone will play an important role in the acceptance and the future development of plasmids for gene therapy, as well as other DNA-based therapeutics. Similar gene therapy treatments based on the tumor suppressor gene p53 are in progress in the United States for refractory head and neck squamous cell cancer (see Table 1). Currently, diseases with complex etiologies such as cancer[23,24] and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease[25] are being targeted. In addition, DNA vaccines for malaria, AIDS, and many other diseases are in development.[26] DNA vaccines have also been used to prevent allergic response.[27]

Oligonucleotides

Mechanism of Action

Oligonucleotides are short single-stranded segments of DNA that upon cellular internalization can selectively inhibit the expression of a single protein.[28] For antisense applications, oligonucleotides interact and form a duplex with the mRNA or the pre-mRNA and inhibit their translation or processing, consequently inhibiting protein biosynthesis (see Figure 1) [28] For antigene applications, oligonucleotides must enter the cell nucleus, form a triplex with the double-stranded genomic DNA, and inhibit the translation as well as the transcription processes of the protein (see Figure 1).[2] Theoretically, because only one target needs to be inhibited in each cell, antigene oligonucleotides should have much better control in protein ablation than antisense oligonucleotides.[2]

Oligonucleotides achieve transcriptional arrest of their target proteins upon interaction with their mRNA targets. Antisense target recognition is facilitated by Watson-Crick base pairing between the oligonucleotide and the target mRNA. On the molecular level, numerous mechanisms have been proposed to explain the basis of oligonucleotide action.[28,29] Occupancy-based mechanisms suggest that binding of oligonucleotides to key sequences in the target may inhibit the target's ability to interact with the cellular machinery required for protein synthesis. For example, oligonucleotide interaction with the initiation codon or with the splicing sequences can cause transcriptional arrest by this route.[28] Alternatively, oligonucleotides can function by activation of RNase H, an enzyme that degrades the targeted mRNA, thereby inhibiting protein biosynthesis.[29]

Since the targets for antisense applications are in the cytoplasm, oligonucleotides do not need to enter the cell nucleus (see Figure 1).[2] However, for antigene applications, oligonucleotides must enter the nucleus to interact with the DNA (see Figure 1). Antigene recognition occurs by formation of DNA-oligonucleotide triplex motifs, which are speculated to function by occupancy-based mechanisms such as physical blockade of the transcription complex (see Figure 1).[2]

Design of Oligonucleotides

Design is critical for the clinical efficacy of oligonucleotides and should include consideration of length, chemistry, conformation, and ability to hybridize with the target mRNA.

Length. The suggested optimal length for oligonucleotides with efficient antisense activity ranges from 12 to 28 bases.[28,30] There is a consensus that very short sequences are likely to be nonspecific and sequences longer than 25 bases would experience difficulty in cellular uptake. As the length of the molecule increases, hydrogen bonding between the base pairs and stacking interactions increases, leading to an increased overall affinity for the target. However, longer oligonucleotides are inherently difficult to import into cells because of their size and tend to self-hybridize, thereby affecting their uptake. Even in cell culture experiments, the optimum length suggested for oligonucleotides varies considerably.[31,32]

Backbone Modifications. Oligonucleotides having the endogenous phosphodiester backbone are susceptible to degradation by nucleases and hence have limited use for antisense applications.[30] Various chemical modifications to the backbone have been used to improve oligonucleotide stability.[33] The most common modifications include the introduction of phosphorothioate and methyl phosphonate linkages in the backbone. Phosphorothioate analogs are chosen for their stability[28] against nucleases and the methylphosphonate backbone for its relative hydrophobicity and ease of diffusion across membranes.[34] Phosphorothioate backbone oligonucleotides can have significantly increased biological half-life compared to their corresponding unmodified phosphodiester oligonucleotides. Currently, second-generation mixed-backbone oligonucleotides such as GEM231 are being used in clinical trials for cancer.[28]

Hybridization Capacity. Antisense oligonucleotides are designed primarily for their ability to hybridize with the mRNA of interest.[35] The mRNA has a complex secondary and tertiary spatial structure that restricts the accessibility of certain segments of the molecule to hybridize with the oligonucleotide. Consequently, although it is theoretically possible to design antisense constructs to virtually any part of the mRNA, not all oligonucleotides are efficient in inhibiting protein synthesis.[36] Complex algorithms predicting mRNA structure, "gene-walking," RNase H mapping, and combinatorial screening are some of the strategies that are being explored to predict hybridization-accessible sites on mRNA molecules.[36]

Secondary Conformation. The presence of some specific sequences in the oligonucleotide can allow it to have a preferred conformation.[37] For example, it was demonstrated that the presence of continuous guanines at the 3'-end of an oligonucleotide made it resistant to degradation because of the ability of these guanines to assemble into hyperstructures in vivo.[38] These hyperstructures were resistant to nuclease activity and were efficiently taken up by cells.[38] The secondary structure of oligonucleotides can be affected by divalent cations such as Ca^{2+} and Mg^{2+} that typically occur in the cellular environment, thereby possibly affecting their activity or cellular uptake.[37]

Clinical Applications of Oligonucleotides

For therapeutic purposes, oligonucleotides can be used to selectively block the expression of proteins that are implicated in diseases.[39] With successful antisense inhibition of pro-

teins in animal models, the first antisense drug, fomivirsen sodium (Vitravene, Isis Pharmaceuticals, Carlsbad, CA) was approved for the treatment of cytomegalovirus retinitis in AIDS patients in 1998.[40] Fomivirsen sodium is formulated as an intravitreal aqueous injection in sodium bicarbonate buffer at pH 8.7.[40] Antisense oligonucleotides such as MG98 and ISIS 5132, designed to inhibit the biosynthesis of DNA methyltransferase and c-raf kinase, respectively, are in human clinical trials for cancer.[41] ISIS 2302, targeting ICAM-1, is being investigated for the treatment of ulcerative colitis[39] (see Table 1). Two other drug candidates, Affinitak and Alicaforsen (Isis Pharmaceuticals), are in phase 3 clinical trials for non-small cell lung cancer and Crohn's disease, respectively. Genasense (oblimersen sodium), developed by Aventis (Bridgewater, NJ) and Genta (Berkeley Heights, NJ), is being investigated in advanced phase 2 trials in combination with other chemotherapy regimens for a range of cancers including malignant melanoma, chronic lymphocytic leukemia, and multiple myeloma. Currently, studies confirming the effects of antigene oligonucleotides are restricted to in vitro applications such as inhibition of bacteriophage T7 transcription initiation and human RNA polymerase II elongation.[2]

Ribozymes

Ribozymes are RNA molecules that are capable of sequence-specific cleaving of mRNA molecules.[2] They can selectively bind to target mRNAs and form a duplex, which includes a highly distorted conformation that is easily hydrolyzed. The hydrolysis of the mRNA can be used for targeted suppression of specific genes.[41] Two types of ribozymes, the hammerhead and hairpin ribozymes, have been extensively studied for therapeutic applications.[2] The presence of the RNA backbone in ribozymes makes them easy targets for degradation from the ubiquitous RNases, so these molecules are biologically unstable in vivo.[41] Ribozymes have been used for gene suppression for apoptosis and antiproliferative effects. Ribozymes can be used for knockout gene therapy by targeting overexpressed oncogenes such as the human epidermal growth factor receptor-Type-2 gene implicated in breast cancer[42] and human papillomavirus virus infection.

DNAzymes

DNAzymes are analogs of ribozymes with greater biological stability.[39] The RNA backbone chemistry is replaced by the DNA motifs that confer improved biological stability. DNA sequences are also easy to modify synthetically, thereby generating even stronger, resilient second-generation analogs. The strong catalytic activity of DNAzymes makes them powerful tools for molecular biology applications such as target validation protocols. DNAzymes have tremendous potential as gene suppression agents for a variety of therapeutic applications. Recently, a DNAzyme directed against the vascular endothelial growth factor receptor 2 was confirmed to be capable of tumor suppression by blocking angiogenesis upon intratumoral injections in mice.[43]

Aptamers

Aptamers are small single-stranded or double-stranded nucleic acid segments that can directly interact with proteins.[2] Aptamer-assisted interaction can be used to interfere with the molecular functions of disease-implicated proteins or those that participate in the transcription or translation processes, thus inhibiting the processing of target therapeutic proteins. Aptamers are preferred over antibodies in protein inhibition owing to their specificity, nonimmunogenicity, and stability of pharmaceutical formulation.[44] Currently, however, most aptamer research is directed toward lead identification of inhibitors of protein function and target validation. Aptamers that have demonstrated promise in intervention of pathogenic protein biosynthesis include DNA aptamers against the HIV-1 integrase enzyme[45] and RNA aptamers against HIV-1 transcriptase.[46] The anti-VEGF aptamer Macugen (pegaptanib sodium) co-developed by Eyetech (New York, NY) and Pfizer (Groton, CT) is in advanced phase 3 clinical trials for age-related macular degeneration to inhibit excessive pathological blood vessel growth in the macula (see Table 1).

Small Interfering RNAs

SiRNAs can be used for downregulation of disease-causing genes through RNA interference. SiRNAs are short double-stranded RNA segments with typically 21- to 23-nucleotide bases that are complementary to the mRNA sequence of the protein whose transcription is to be blocked.[47-49] Upon administration, siRNA molecules are incorporated into RNA-induced silencing complexes (RISCs), which bind to the mRNA of interest and stimulate mRNA degradation mechanisms, such as nuclease activity, that lead to silencing of the particular gene.[47-49] The structure and functions of RISCs have yet to be completely elucidated.[50] SiRNA molecules used for protein ablation can either be chemically synthesized and delivered to the cells or generated in vivo.[50] In vivo production of siRNAs is achieved by using long double-stranded RNA molecules, which are processed by the Dicer enzyme to generate siRNAs, or by transfection with plasmids and viral vectors that biosynthesize siRNAs by transcription.

The use of siRNA as a therapeutic target is still in its infancy. Currently, siRNAs are being investigated to inhibit HIV,[51] hepatitis,[52] and influenza infection.[53] These potent molecules offer several advantages over plasmid DNA molecules and oligonucleotides. Compared with other gene ablation technologies such as antisense oligonucleotides, siRNAs are remarkably superior thanks to their high degree of specificity to mRNAs, their nonimmunogenic nature, and their high resistance to

ribonucleases.[47] Since siRNAs do not integrate into the genome, they offer greater safety than plasmid molecules. Furthermore, siRNAs do not have to transfer through the nuclear membrane for their activity and therefore require less sophisticated delivery systems, promising faster development and higher efficiencies compared with plasmid DNA. In addition, because of their small size, it might be possible to deliver a cocktail of siRNAs targeting multiple disease-causing genes in a single delivery system to control complex diseases such as cancer where several genes are malfunctioning.[54]

CELLULAR UPTAKE OF DNA-BASED THERAPEUTICS

Figure 1 is an interactive and animated schematic representation of the delivery, uptake, and intracellular fate of DNA-based therapeutics. The reader can access the user-controlled interface, select the type of DNA-based therapeutic, and observe the graphic animation of its uptake and subsequent intracellular fate, as described below for individual classes of therapeutics. The cellular uptake of naked DNA molecules and polynucleotides in general, irrespective of their size, remains an extremely inefficient process. A combination of several inherent factors such as DNA charge, size, and poor stability presents a potent barrier to cellular uptake (see Figure 1). The negatively charged phosphate backbone of the DNA molecule is the primary cause of its inadequate and inefficient cellular association, owing to electrostatic repulsion from the negatively charged cell surface. Delivery of plasmid DNA is further reduced by its high molecular weight compared with shorter DNA therapeutics such as oligonucleotides and ribozymes. In addition to their inherently ineffective cellular uptake profile, chemically unmodified or naked DNA therapeutics have extremely low in vivo stability. They are rapidly degraded by hydrolytic endo- and exonucleases. Nucleases recognize the phosphodiester linkage in the DNA backbone and can lead to hydrolytic degradation of the DNA molecule. Despite these factors, it has been clearly demonstrated not only that naked DNA can be internalized by muscle cells but also that it can retain its complete functionality and can express proteins.[55] For example, plasmid DNA containing the transgene for the influenza protein was demonstrated to stimulate protective immunity in mice upon administration without any delivery system.[55]

It is generally believed that DNA molecules that are associated with the cell membrane are internalized into the cytoplasm by cell surface receptors via receptor-mediated endocytosis[55,56] (see Figure 1). Over the past several decades, putative DNA receptors for plasmids and oligonucleotides have been identified and characterized.[56,57] These studies have provided valuable insights into DNA uptake, but no universal mechanisms have been conclusively identified, further exemplifying the complexity of the process and the possibility of tissue- and cell-specific receptors. Other processes such as pinocytosis and absorptive endocytosis may also play an important role in oligonucleotide uptake.[58] Recent work by Huang and coworkers demonstrated that naked plasmid DNA can be internalized by lung endothelial cells if sufficient time is allowed for the DNA to interact with its putative receptor.[59] This finding has raised the interesting possibility of dependence of DNA cellular uptake on the time of contact between the DNA molecule and its target receptor.[59]

The small fraction of the DNA therapeutics that does obtain cellular access is susceptible to degradation in the endosomes (see Figure 1). Upon endocytotic internalization, DNA and other polynucleotides, along with their delivery vectors, are compartmentalized into endosomal vesicles, where the DNA can be inactivated or degraded.[60-62] The endosome undergoes acidification to a pH of 5 to 6, which, in addition to promoting acidic hydrolysis, activates lysosomal enzymes that can rapidly degrade oligonucleotides or plasmids.[60,61] If the DNA therapeutic escapes from the endosome and degradation therein, it can enter the cytosol and may have a chance to assert its pharmacological effect (see Figure 1).[58,59] Traffic of DNA-based therapeutics such as antisense oligonucleotides and ribozymes is restricted to the cytoplasm, where their molecular targets (mRNA) are located.[58] However, plasmids and antigene oligonucleotides require nuclear entry to be therapeutically active (see Figure 1).[58,63] The prerequisite of nuclear entry poses an additional challenge to achieving successful clinical outcomes.

Transfer of macromolecules into the nucleus is controlled by the pores in the nuclear membrane (see Figure 1).[63,64] Oligonucleotides, by virtue of their smaller size, can passively diffuse from the cytoplasm into the nuclear compartment relatively easily. However, very little is known about plasmid DNA nuclear transfer and the factors that influence it.[63] Plasmid DNA undergoes dissociation from its delivery vector and can access the nucleus either during mitosis, when the nuclear envelope is weakened, or through the nuclear pores.[65,66] Other nonmitotic mechanisms have also been proposed.[63]

If plasmid DNA does enter the nucleus, it can integrate with the host genome and use the cellular machinery to express the transgene of interest (see Figure 1). Depending upon the vector used to deliver them, plasmid DNA molecules may demonstrate high or low and site-specific or nonspecific genomic integration with the host cell. However, genomic integration may provoke other problems, such as mutagenesis and carcinogenesis.[63]

STRATEGIES TO ENHANCE THE CELLULAR DELIVERY OF DNA-BASED THERAPEUTICS

From an evolutionary perspective, it is to our advantage that transfer of DNA molecules across cellular barriers remains dif-

ficult, thereby preserving our genomic information and protecting us from genetic contamination.[3] It is evident that the cellular environment is unfavorable to the entry of DNA-based therapeutics and detrimental to their intracellular trafficking and nuclear transfer. Thus, the overall inefficiency of the cellular internalization process jeopardizes the complete exploitation of the benefits of DNA-based therapeutics. To realize the efficacy of DNA therapeutics, the natural barriers to DNA transport must be either evaded or inactivated (see Figure 1).

Tremendous strides have been made in developing strategies for DNA delivery into cells, both in vitro and in vivo, that can protect it from degradation and facilitate targeted cellular uptake. The choice of strategy is determined by the DNA-based therapeutic of interest and its final clinical outcome (see Figure 1). Nuclease degradation of oligonucleotides and other shorter DNA therapeutics can be circumvented by chemical derivatization of the backbone and/or by the protection and stability offered by DNA delivery systems. Plasmid DNA molecules, however, rely exclusively on the latter. For example, mixed-backbone oligonucleotides containing methylphosphonate linkages have exhibited significantly improved biological half-lives compared with their phosphorothioate analogs.[67] The biological half-life of 1.07 hours of an unencapsulated oligonucleotide ISIS 2302 was improved to 57.8 hours by encapsulation into pegylated stealth liposomes.[68] Similarly, plasmid DNA half-life was increased to more than 6 hours when it was administered in the form of stabilized plasmid-lipid particles.[69]

In addition to ensuring the stability of DNA-based therapeutics, it is essential to ensure their rapid escape and protection from the endosomal degradation (see Figure 1). This has been achieved by the use of viral delivery vectors or by the inclusion of fusogenic lipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) in pH-sensitive and cationic liposome delivery systems.[70,71] DOPE is a helper lipid capable of disrupting the endosomal membrane upon endosomal acidification by the formation of lipid hexagonal phases. Endosomal membrane disruption can release the DNA-based therapeutic and its delivery system into the cytoplasm.[71] Lysosomatropic agents such as monensin and chloroquine, which raise the endosomal pH, block acidification, and thus inhibit lysozyme activity, have also been used to facilitate endosomal release of DNA.[60,61] Endosomal degradation of DNA-based therapeutics can also be circumvented by the incorporation of viral peptides such as hemagglutinin HA2 and those derived from adenoviruses in their delivery systems.[63,65,66] Hemagglutinin HA2 undergoes conformational transition and leads to the destruction of the endosome, thereby facilitating the release of the DNA-based therapeutic.[63] Enhanced rapid endosomal escape and enhanced transfection have also been achieved using fusogenic peptides such as poly(L-lysine) (PLL) and cationic polymers such as polyethylenimine (PEI) and dendrimers.[63,65,66]

The need for nuclear delivery of DNA-based therapeutics is limited to plasmids, which must integrate with the host genome (see Figure 1). Plasmid nuclear uptake has been enhanced by its binding to nuclear localization signal peptides, transcriptional factors (eg, GAL4, SV-40, and SMGA), histones, and by the use of peptide nucleic acid constructs.[6,63] Several cationic delivery vectors can significantly compact DNA to facilitate its passage across nuclear membranes, thus leading to successful transfection.[72-74] Recently, novel extranuclear cytoplasmic expression vectors have been developed that totally circumvent the need for nuclear access by expressing plasmids in the cytoplasm.[65] This route uses cytosolic cellular machinery for transgene expression.[65]

DNA Delivery Techniques

DNA delivery methods can be classified into 3 general types: electrical techniques, mechanical transfection, and vector-assisted delivery systems. Since this review primarily focuses on various platforms that have been developed for the delivery of DNA-based therapeutics, we have briefly summarized some of the major advantages and disadvantages of the electrical and mechanical techniques in a single section. The reader is recommended to refer to the cited reviews in this section for a more detailed discussion on these techniques.

Mechanical and Electrical Techniques

Mechanical and electrical strategies of introducing naked DNA into cells include microinjection,[75] particle bombardment,[66] the use of pressure,[66] and electroporation.[76] Microinjection is highly efficient since one cell at a time is targeted for DNA transfer; however, this precision is achieved at the expense of time.[66] Ballistic transfer of DNA-gold microparticles can be achieved using particle bombardment equipment such as the gene gun.[66,77] However, since direct exposure of target tissues is required, particle bombardment is restricted to local expression in the dermis, muscle, or mucosal tissue, unless surgical exposure of the target is permitted.[66] Electroporation uses high-voltage electrical current to facilitate DNA transfer. This technique results in high cell mortality and therefore is not suitable for clinical use. Though significant transfection efficiencies have been achieved using mechanical and electrical techniques, they are extremely difficult to standardize in a clinical setting and are considered laborious, impractical, and invasive.[66]

Vector-Assisted Delivery Systems

Cellular delivery of DNA assisted by delivery systems has matured from a laboratory science into a methodology suitable for use in clinical trials of DNA-drug candidates (see Table 1).[58,59,66] Delivery systems will play a critical role in the realization of the therapeutic potential of DNA therapeu-

tics. Some of the ideal properties in a DNA delivery vector for therapeutic purposes include high transfection efficiency with a high degree of target cell specificity, low occurrence of toxicity and immunogenicity, biodegradability, and stability of the pharmaceutical formulation. In addition, the ideal delivery system would be simple to formulate and would lend itself to easy modification for customized DNA release, delivery, and expression. Currently available DNA delivery systems can be classified into 2 types based on their origin: biological viral DNA delivery systems and chemical nonviral delivery systems.

Viral Delivery Systems

Nonpathogenic attenuated viruses can be used as delivery systems for DNA molecules, especially plasmids.[63,78,79] Through millions of years of evolution as infective agents, viruses can transfer DNA molecules into cells with consummate ease. For therapeutic purposes, the transgene of interest is assembled in the viral genome and the virus uses its innate mechanism of infection to enter the cell and release the expression cassette. The gene then enters the nucleus, is integrated into the host gene pool, and is eventually expressed.[63] Retroviruses,[80] parvoviruses, adenoviruses, lentiviruses,[81] adeno-associated viruses,[82] and the herpes simplex virus are being investigated for their ability to transfer DNA. Gene expression using viral vectors has been achieved with high transfection efficiencies in tissues such as kidney,[83] heart, muscle,[84] eye,[82] and ovary.[85] Viruses are currently used in more than 70% of human clinical gene therapy trials worldwide.[86] Gene therapy using viral systems has made considerable progress for the treatment of a wide range of diseases, such as muscular dystrophy,[84] AIDS,[87] and cancer.[88] The only approved gene therapy treatment (Gendicine) delivers the transgene using a recombinant adenoviral vector.[22] DNA delivery using viral vectors has been extensively reviewed.[78,79,89]

One significant advantage of viral DNA vectors is their extremely high transfection efficiency in a variety of human tissues. Retroviral vectors are capable of transfecting high populations (45%-95%) of primary human endothelial and smooth muscle cells, a class of cells that are generally extremely difficult to transfect.[90] A recent study demonstrated that adenoviral-mediated gene transfer in COS-7 cells was significantly higher than that achieved by liposomal delivery systems.[91]

Despite such impressive statistics for gene transfer, there are several concerns over the use of viruses to deliver DNA therapeutics in humans. The chief concern is the toxicity of the viruses and the potential for generating a strong immune response owing to their proteinaceous capsid. Such toxicities have been observed in numerous animal models.[92-94] For example, it was demonstrated that administration of recombinant adeno-associated virus to macaques, a nonhuman primate model, at doses similar to those administered to hemophilia B patients undergoing gene therapy, caused transient viremia.[93] Viral metabolite sequences were identified in lymph nodes and livers as long as 8 to 18 months after viral injection. The long-term effects of viral artifacts on body organs and primate health are not yet known. Another study demonstrated local inflammation at the site of injection upon intraperitoneal administration of an adenoviral vector formulation in mice used as a preclinical model for prostate cancer. At high doses, these vectors were detected in seminal vesicles, testes, and the lymph nodes.[94] These results contradicted earlier findings that had indicated that germ line transmission of viral vector sequences was very low in several species of male animals.[95] Additionally, since animal studies are generally performed under tightly regulated conditions such as specific strains and regimented nutrition, it is difficult to use these results to predict human response in a diverse patient population.

Safety of viruses is a major concern in human studies. Recently, traces of adenovirus titers were detected in seminal fluids of a male patient who had earlier received adenoviral-based genetic therapy, further compounding the fear of possible germ line tampering by viruses upon loss of their replication deficiency.[96] Adenoviral vectors used for gene therapy for cystic fibrosis were shown to cause a strong immunogenic response.[97] The 1999 death of a patient participating in a Food and Drug Administration-approved gene therapy clinical trial from respiratory and multiple organ failure, attributed to lethal immune response to the adenovirus vector used to deliver the gene, led to temporary suspension of all gene therapy trials in the United States.[98] Though clinical experimentation has resumed, this event raised tremendous concern over the safety of viruses for future gene therapy applications.[98] Additionally, the integration of therapeutic genes into the host genome by the virus takes place in a random fashion. There is no control over the exact location of insertion of the gene. Random gene transfer can generate insertional mutagenesis that may inhibit expression of normal cellular genes or activate oncogenes, with deleterious consequences.

Many other factors may limit the use of viral vectors for therapeutic applications. Since the viral envelope has a finite capacity, there is a limit on the size of the expression plasmid that it can incorporate. First generation adeno-associated viruses had a very small capacity of ~4.7 kilobase (kb) for encapsulation of the plasmid DNA cargo.[99] Recent reports demonstrate efficient production of second-generation adeno-associated viruses with higher encapsulating capabilities.[99]

It has been demonstrated that adenoviruses in formulations may lose their potency after storage in commonly used pharmaceutical vials.[100] Adenoviral vectors are known to gener-

ate inflammatory responses in tissues with short-lived gene expression.[77] There is concern over loss of transgene expression upon tissue maturation when cells have been transfected using viruses.[101] For example, retroviral vectors have been demonstrated to lose long-term expression capability in newborn Swiss mice, limiting their potential clinical applications.[102] Retroviral vectors for gene therapy applications are also extremely difficult to produce on a large scale and are inherently unstable.[80] Production of viral titers is expensive, with complicated scale-up issues. It will be very difficult to use viruses for routine gene transfer until all of these concerns are addressed.

Nonviral Delivery Systems

Nonviral delivery systems can circumvent some of the problems associated with viral vectors and are emerging as favorable alternatives to viral vectors. Among the greatest advantages of nonviral gene vectors are lack of immune response and ease of formulation and assembly.[41] Commonly used nonviral vectors for delivery of DNA-based therapeutics can be classified into 2 major types based on the nature of the synthetic material: i) Polymeric delivery systems (DNA-polymer complexes) and ii) Liposomal delivery systems (DNA entrapped in and/or complexed to liposomes).[41,103-105]

Polymeric Delivery Systems

Cationic polymers are commonly used in gene delivery because they can easily complex with the anionic DNA molecules.[106] Polymer-DNA complexes, also known as polyplexes, can be used to deliver DNA into cells. The general mechanism of action of polyplexes is based on the generation of a positively charged complex owing to electrostatic interaction of these cationic polymers with anionic DNA.[66] The cationic polyplex can then interact with the negatively charged cell surface to improve DNA uptake. Versatility of physicochemical properties and easy manipulation are some of the most important advantages of polymeric gene carriers.[41] Polymeric matrices with varying properties can be designed by choosing an appropriate distribution of different molecular weights and degree of cross-linking of the polymer, and/or by the incorporation of targeting ligands.[41] Industrial-scale manufacturing is feasible at low cost. Commonly used polymers include PEI,[107] PLL,[108] chitosans,[109] and dendrimers.[41] Agents such as folates, transferrin, antibodies, or sugars such as galactose and mannose can be incorporated for tissue targeting.[41]

Polymeric transfection systems have advantages, but they are not ideal. In addition to low transfection capabilities, polymeric delivery systems suffer from problems in the control of molecular weight distributions, dispersities of the polyplexes, and other quality control issues.[41] Some polymers have inherent potent pharmacological properties (such as hypocholesterolemia induced by chitosans) that make them extremely unfavorable for human use.[110]

Polyethylenimine

PEI is a branched polymer with high cationic potential that is capable of effective gene transfer in nondifferentiated COS-1 cells; however, it can also be extremely cytotoxic due to induction of apoptosis.[111] The high transfection efficiency of PEI can be attributed to the buffering effect or the "proton sponge effect" of the polymer caused by the presence of amino groups in the molecule.[63] The strong buffering effect of the polymer helps in rapid endosome escape.[112] The cytotoxicity and transfection efficiency of PEI are directly proportional to its molecular weight.[113] Efforts to reduce the toxicity by synthesis of PEI with graft copolymers such as linear poly(ethylene glycol),[114] incorporation of low molecular weight PEI, and PEI glycosylation[115] are under way.

Poly(L-lysine)

PLL is a biodegradable cationic polymer that has been used to deliver DNA-based therapeutics such as oligonucleotides.[41] Cationic PLL interacts with anionic DNA molecules and forms a positively charged complex that can interact with the negatively charged cell surface and undergo rapid internalization.[41] However, PLL has a low level of transfection efficiency, primarily owing to lack of rapid release of PLL-DNA complexes from endosomes. It was recently established that PLL-DNA complexes undergo biodistribution into acidic lysosomes that favor DNA degradation upon cellular internalization.[116] PLL suffers from immunogenicity and toxicity caused by its amino acid backbone.[117] PLL-DNA complexes also undergo nonspecific binding to cell membranes, thereby limiting their use in cell targeting.[118] Consequently, PLL has been used in gene delivery as an adjunct to graft copolymers and other endosomolytic agents to improve their uptake and targeting. For example, it was demonstrated that PLL-poly(lactic-co-glycolic) acid (PLGA) grafted micelles had high transfection efficiencies and low cytotoxicities compared with PLL alone.[119] PLL transfection capacity was significantly improved by pretreating the PLL-DNA complex with peptides and an endosomolytic agent such as chloroquine.[120] Targeting of PLL to liver was significantly improved by its conjugation to a liver tissue-specific ligand, asialoorosomucoid.[65] From a formulation perspective, PLL-DNA complexes demonstrated a high degree of polydispersity, leading to unreliable cellular DNA delivery.[66]

Chitosan

Chitosan is a natural biodegradable polymer that is a possible alternative to PEI owing to its low toxicity.[112,121] Like

PLL, chitosan generates a positively charged complex upon interaction with DNA that favors its cellular interaction and subsequent uptake. The transfection efficiency of chitosan is, however, lower than that of other polymers such as PEI, as observed by a comparison study consisting of intratracheal administration of chitosan-DNA polyplexes to mice in vivo.[112] Chitosan has been used in nonconventional gene delivery. For example, chitosan was recently used to augment the in vitro infectivity of an adenoviral vector in Chinese hamster ovary cells.[122] Topical application of chitosan microparticles containing the luciferase gene has been demonstrated to express the reporter gene in mouse skin, suggesting a potential use of chitosan for genetic immunization.[123] Chitosan-DNA nanoparticles, upon oral administration, were capable of conferring immunological protection to mice against a peanut antigen, indicating a possible use of chitosan in delivering genetic vaccines to control food allergens.[124] However, chitosan at high doses can cause hypocholesterolemia in humans, thereby limiting its applications.[110]

Dendrimers

Polyamidoamine (PANAM) dendrimers represent a novel class of polycationic synthetic polymers that can be used for gene transfer.[58,125,126] The 3-dimensional spherical structure of dendrimers offers synthesis control of the molecule in terms of degree and generation of branching. This control can produce polymer particles with a very low degree of polydispersity, which is a significant advantage over other polymers such as PLL that generate highly polydisperse particles. Low polydispersity can lead to reproducible gene delivery and a clinically reliable formulation. The cationic amino acid residues in the polymeric structure of PANAM dendrimers can help in DNA condensation and endosome release.[41] Superfect and Polyfect (Qiagen, Valencia, CA) are 2 commercially available sixth-generation branched activated dendrimer formulations for in vitro gene delivery.[41] Some reports suggest that lower generations also can be effective in gene delivery.[127] Dendrimers that protect oligonucleotides from serum nucleases[39] have been used to enhance oligonucleotide delivery.[128]

Liposomal Delivery Systems

Liposomes have emerged as one of the most versatile tools for the delivery of DNA therapeutics.[39,103,104,113] Liposomes are vesicles that consist of an aqueous compartment enclosed in a phospholipid bilayer. If multiple bilayers of lipids are formed around the primary core in a concentric fashion, the complex assemblies that are generated are known as multilamellar vesicles (MLV). MLV can be formed by reconstituting thin lipid films in buffer. Small unilamellar vesicles (SUV) of specific size (100-500 nm) can be produced by extruding MLV through polycarbonate membranes. SUV (50-90 nm) can also be produced by sonication of MLV or larger SUV. Both hydrophilic and hydrophobic drugs can be entrapped in the liposomes. Liposomes and drug/lipid complexes have been used for the delivery of the anticancer drugs doxorubicin and daunorubicin.[129]

Liposomes can be used as DNA drug delivery systems either by entrapping the DNA-based therapeutics inside the aqueous core or complexing them to the phospholipid lamellae. Liposomes offer significant advantages over viral delivery options for the delivery of DNA therapeutics. For example, liposomes are generally nonimmunogenic owing to lack of proteinaceous components. Since the phospholipid composition in the liposome bilayers can be varied, liposomal delivery systems can be easily engineered to yield a desired size, surface charge, composition, and morphology. The cationic surface charge of some liposomes can help in the complexation and delivery of DNA therapeutics. Liposomes can offer substantial protection to the DNA therapeutics from nucleases and improve their biological stability. Liposomes can also be used for specialized gene delivery options (such as long circulation half-life, sustained, and targeted delivery).[104] A variety of cationic, anionic, synthetically modified lipids, and combinations thereof have been used to deliver a wide range of DNA-based therapeutics.

Cationic Liposomes. Numerous studies have demonstrated the use of cationic liposomal formulations for the delivery of different plasmid constructs in a wide range of cells, both in vivo and in vitro.[130] The nonimmunogenic nature and ease of industrial production of these systems makes them appealing for gene transfer. The first human gene therapy trial using cationic liposomes was conducted in 1992.[77] The trial used 3ß[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol)/DOPE cationic liposomes to deliver the transgene of interest. Currently, ~13% of gene therapy trials in progress worldwide employ nonviral liposomal vectors for transgene delivery.[41]

Cationic liposomal formulations generally consist of mixtures of cationic and zwitterionic lipids.[113,131,132] Cationic lipids commonly used are 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), N-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), 2,3-dioleoyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium (DOSPA), dioctadecyl amido glycil spermine (DOGS), and 3,[*N*-(*N*^{1},*N*-dimethylethylenediamine)-carbamoyl]cholesterol (DC-chol).[130] Commonly used zwitterionic lipids, also known as helper lipids, are DOPE and cholesterol.[130] The cationic lipids in the liposomal formulation serve as a DNA complexation and DNA condensation agent during the formation of the lipoplex. The positive charge also helps in cellular association. The zwitterionic lipids help in membrane perturbation and fusion. Proprietary formulations of cationic lipids such as Lipofectamine (Invitrogen,

Carlsbad, CA), Effectene (Qiagen, Valencia, CA), and Tranfectam (Promega, Madison, WI) are commercially available,[133] but most of the transfection kits are useful only for in vitro experimentation.

Despite the appreciable success of cationic lipids in gene transfer, toxicity is of great concern. Cytotoxicity of cationic lipids has been established in numerous in vitro[134,135] and in vivo[136,137] studies. Using a battery of cell proliferation assays (such as [^{3}H]thymidine incorporation) and cytotoxicity tests (such as lactate dehydrogenase release upon cell lysis), it was demonstrated that cationic liposomes composed of dimethyldioctadecyl ammonium bromide (DDAB)-DOPE and the commercial transfection-reagent DOTAP were cytotoxic to CaSki cells, a human cervical cancer cell line.[134] Cationic liposomes were also confirmed to cause dose-dependent pulmonary toxicity in mice upon intratracheal instillation.[136] The study demonstrated that cationic liposomes stimulated the production of reactive oxygen intermediates that have been previously implicated in pulmonary toxicity.[136] Cationic lipids have also been demonstrated to be cytotoxic to phagocytic macrophages.[137] Reduction in toxicity was observed upon the replacement of DOPE by 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), possibly suggesting the role of the helper lipid DOPE in the toxicity of cationic liposomes.[137] In other studies, cytokine-mediated pulmonary toxicity and TNF-α induction by cationic lipids in lung tissue have been identified.[138]

Additionally, the transfection efficiencies of nonviral cationic liposomal vectors are significantly lower than those of viral vectors. Low transfection efficiencies have been attributed to the heterogeneity and instability of cationic lipoplexes.[139] Dissimilarity in lipoplex size also adversely affects their quality control, scale-up, and long-term shelf stability, issues that are pertinent to their pharmaceutical production. Another drawback in the use of cationic lipids is their rapid inactivation in the presence of serum.[132,140] It was demonstrated that transient complexes formed between cationic liposomes and plasmid DNA prior to transfection were completely inactivated when experiments were performed in the presence of 2% fetal calf serum.[132] Similarly, Lipofectin-mediated transfection was inactivated by serum.[140] In addition, most of the in vitro studies that demonstrate the transfection efficacy of cationic lipid-based systems were conducted in standardized media formulations such as Opti-MEM (Invitrogen) that do not contain serum. Therefore, in vitro/in vivo correlation is misleading and unreliable. Some in vivo studies have revealed that the gene transduction responses obtained by cationic liposomes were transient and short-lived.[141,142] Routine transfer of genetic drugs using cationic lipids is impossible without the elimination of these obstacles. Novel synthetic strategies and formulation approaches may yield progress in this direction.

Anionic Liposomes. As an alternative to cationic lipids, the potential of anionic lipids for DNA delivery has been investigated.[3,37,135,143-146] The safety of anionic lipids has been demonstrated when administered to epithelial lung tissue.[136] In recent years, a few studies[143,144] using anionic liposomal DNA delivery vectors have been reported. They include the delivery of oligonucleotides to hippocampal neurons using anionic liposomes.[144] Similarly, an anionic lipid formulation called fluid liposomes, composed of DPPC and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG), was capable of delivering fluorescently labeled oligonucleotides into bacterial cells.[143] In this case, the entrapment of oligonucleotides within the liposomes was facilitated by Na^+ and K^+ ions. There have been attempts to incorporate anionic liposomes into polymeric delivery systems.[147,148] For example, LPDII vectors are nonviral vehicles for gene delivery that consist of a complex between anionic pH-sensitive liposomes and polycation-condensed plasmid DNA (polyplexes).[147,148] However, these vectors have limited applications, mainly because of (1) inefficient entrapment of DNA molecules within anionic liposomes[144] and (2) lack of toxicity data.[143] Lack of further progress of these systems may be attributed, in part, to the poor association between DNA molecules and anionic lipids, caused by electrostatic repulsion between these negatively charged species.[37,149] We have recently reported the development of a novel anionic lipoplex delivery system with high transfection efficiency and low toxicity.[3,37,135,145,146] This vector is composed of a mixture of anionic lipid 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) and zwitterionic lipid DOPE. These lipids are known to occur endogenously in vivo and are complexed to DNA molecules using divalent Ca^{2+}.[3,146] The resulting ternary complex of lipids, calcium ions, and DNA was shown to be capable of transfecting mammalian cells with a model green fluorescence reporter protein.[146]

Specialized Liposomal Delivery Platforms. Along with numerous cationic and anionic lipid derivatives, functionalized liposomal formulations serving specific therapeutic objectives have shown promise in gene therapy.[103,150,151] Liposomes that contain targeting and functionalizing groups in their lipid bilayers can be tailor-made for a range of specialized DNA delivery options.[150,151] These strategies also offer potential options in the development of sophisticated delivery platforms for DNA drugs. Specialized liposomal delivery platforms include pH-sensitive liposomes, immunoliposomes, and stealth liposomes.

pH-Sensitive Liposomes. pH-sensitive liposomes can be generated by the inclusion of DOPE into liposomes composed of acidic lipids such as cholesterylhemisuccinate or oleic acid.[150] At the neutral cellular pH 7, these lipids have the typical bilayer structure; however, upon endosomal compartmentalization they undergo protonation and collapse into a nonbilayer structure, thereby leading to the disruption and destabilization of the endosomal bilayer, which in turn helps in the rapid release of DNA into the cytoplasm.[150] Efficient

gene delivery of the beta-galactosidase and luciferase reporter plasmids has been obtained using pH-sensitive liposomes in a variety of mammalian cell lines.[152] Recently, a chemical derivative of DOPE, Citraconyl-DOPE, has been used to deliver DNA-based therapeutics to cancer cells, thereby combining the targeting and the rapid endosome-releasing aspects of specialized liposomal delivery systems.[153] The researchers reported that small amounts of 3% Citraconyl-DOPE could be used for transfection and the pH-sensitive effect.[153] A phosphatidylcholine/glycyrrhizin combination was also successful in pH-sensitive gene delivery in mice.[154]

Immunoliposomes. Immunoliposomes are sophisticated gene delivery systems that can be used for cell targeting by the incorporation of functionalized antibodies attached to lipid bilayers.[151] Immunoliposomes target specific receptors and facilitate receptor-mediated endocytosis for the uptake of the lipoplex. Recently, immunoliposomes containing an antibody fragment against the human transferrin receptor were successfully used in targeted delivery of tumor-suppressing genes into tumors in vivo.[155] Tissue-specific gene delivery using immunoliposomes has been achieved in the brain,[156] embryonic tissue,[157] and breast cancer tissue.[158]

Stealth Liposomes. Stealth liposomes are sterically stabilized liposomal formulations that include polyethylene glycol (PEG)-conjugated lipids.[103] The lipids with covalently attached PEG can be included in the formulation at a desired ratio. Pegylation prevents the opsonization and recognition of the liposomal vesicles by the reticuloendothelial system.[103] Consequently, stealth liposomes have long circulating times in the systemic circulation. They have been used in the delivery of oligonucleotides directed against the Ha-Ras oncogene in a primate model.[68] Peglyation has also been used in conjunction with other polymeric delivery systems such as PLL to achieve longer circulation half-lives.[159]

CONCLUSION

Favorable results in recent clinical trials have renewed interest in DNA-based therapeutics. The transition of these novel molecules into drugs of choice for the treatment of complex disorders is still in its infancy but will eventually be achieved by the development of novel DNA delivery platforms that prevent their degradation and facilitate targeting to specific tissues. Simultaneous advances in chemical synthesis and derivatization to generate molecules with robust biological stability will ensure the in vivo success of short-length DNA-based therapeutics such as oligonucleotides, ribozymes, and their next generation analogs. The use of high-throughput systems for lead identification, validation, and optimization of newer disease targets will provide a tremendous impetus in developing newer potent molecules with specific activity. Further advances in studying gene function and identification of single-nucleotide polymorphisms will not only help in fine-tuning DNA-based therapeutics for specific disease variations but will also fulfill the ultimate goal of providing tailor-made individualized medicines that meet universal therapeutic demands.

ACKNOWLEDGEMENTS

The authors would like to thank Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT), for its generous support of a predoctoral graduate fellowship for Siddhesh D. Patil.

REFERENCES

1. Crooke ST. An overview of progress in antisense therapeutics. *Antisense Nucleic Acid Drug Dev.* 1998;8:115-122.

2. Stull RA, Szoka FC Jr. Antigene, ribozyme and aptamer nucleic acid drugs: progress and prospects. *Pharm Res.* 1995;12:465-483.

3. Patil SD, Burgess DJ. DNA-based Biopharmaceuticals: therapeutics for the 21st Century. *AAPS Newsmagazine.* 2003;6 (12):27.

4. Baker BF. The role of antisense oligonucleotides in the wave of genomic information. *Nucleosides Nucleotides Nucleic Acids.* 2001;20(4-7):397-399.

5. Van Ommen GJB, Bakker E, Den Dunnen JT. The human genome project and the future of diagnostics, treatment, and prevention. *Lancet.* 1999;354(suppl 1):5-10.

6. Uherek C, Wels W. DNA-carrier proteins for targeted gene delivery. *Adv Drug Deliv Rev.* 2000;44(2-3):153-166.

7. Johnston SA, Talaat AM, McGuire MJ. Genetic Immunization What's in a Name? *Arch Med Res.* 2002;33:325-329.

8. Denny WA. Prodrugs for gene-directed enzyme-prodrug therapy (suicide gene therapy). *J Biomed Biotechnol.* 2003;1:48-70.

9. Denny WA, Wilson WR. The design of selectively-activated anti-cancer prodrugs for use in antibody-directed and gene-directed enzyme-prodrug therapies. *J Pharm Pharmacol.* 1998;50:387-394.

10. Connors TA. The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. *Gene Ther.* 1995;2:702-709.

11. Bentires-Alj M, Hellin A-C, Lechanteur C, et al. Cytosine deaminase suicide gene therapy for peritoneal carcinomatosis. *Cancer Gene Ther.* 2000;7:20-26.

12. Anderson WF. Prospects for human gene therapy. *Science.* 1984;226:401-409.

13. Fitzsimons HL, Bland RJ, During MJ. Promoters and regulatory elements that improve adeno-associated virus transgene expression in the brain. *Methods.* 2002;28(2):227-236.

14. Walther W, Stein U. Cell type specific and inducible promoters for vectors in gene therapy as an approach for cell targeting. *Journal of Molecular Medicine (Berlin).* 1996;74:379-392.

15. Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum Gene Ther.* 1997;8:2019-2029.

16. Paillard F. Promoter attenuation in gene therapy: causes and remedies. *Hum Gene Ther.* 1997;8:2009-2010.

17. Weeratna RD, Wu T, Efler SM, Zhang L, Davis HL. Designing gene therapy vectors: avoiding immune responses by using tissue-specific promoters. *Gene Ther.* 2001;8:1872-1878.

18. Greco O, Marples B, Dachs GU, Williams KJ, Patterson AV, Scott SD. Novel chimeric gene promoters responsive to hypoxia and ionizing radiation. *Gene Ther.* 2002;9:1403-1411.

19. Anderson WF. Human gene therapy. *Nature (London).* 1998;392(suppl 6679):25-30.

20. Vorburger SA, Hunt KK. Adenoviral gene therapy. *Oncologist.* 2002;7:46-59.

21. Otsu M, Candotti F. Gene therapy in infants with severe combined immunodeficiency. *BioDrugs.* 2002;16:229-239.

22. China OKs Gene Therapy Drug. *Genetic Engineering News.* 2003:6.

23. Galanis E, Russell S. Cancer gene therapy clinical trials: lessons for the future. *Br J Cancer.* 2001;85:1432-1436.

24. Mulherkar R. Gene therapy for cancer. *Curr Sci.* 2001;81:555-560.

25. Baekelandt V, De Strooper B, Nuttin B, Debyser Z. Gene therapeutic strategies for neurodegenerative diseases. *Curr Opin Mol Ther.* 2000;2:540-554.

26. Bunnell BA, Morgan RA. Gene therapy for HIV infection. *Drugs Today.* 1996;32:209-224.

27. Horner AA, Van Uden JH, Zubeldia JM, Broide D, Raz E. DNA-based immunotherapeutics for the treatment of allergic disease. *Immunol Rev.* 2001;179:102-118.

28. Crooke ST. Molecular mechanisms of action of antisense drugs. *Biochim Biophys Acta.* 1999;1489:31-43.

29. Crooke ST. Molecular mechanisms of antisense drugs: human RNase H. *Antisense Nucleic Acid Drug Dev.* 1999;9:377-379.

30. Engels JW, Uhlmann E. Chemistry of Oligonucleotides. In: Malvy C, ed. *Pharmaceutical Aspects of Oligonucleotides.* London, UK: Taylor & Francis Ltd; 2000:35-78.

31. Khatsenko O, Morgan R, Truong L, et al. Absorption of antisense oligonucleotides in rat intestine: effect of chemistry and length. *Antisense Nucleic Acid Drug Dev.* 2000;10:35-44.

32. Stein CA, Subasinghe C, Shinozuka K, Cohen JS. Physicochemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res.* 1988;16:3209-3221.

33. Matteucci M. Structural modifications toward improved antisense oligonucleotides. *Perspectives in Drug Discovery and Design.* 1996;4(Antisense Therapeutics):1-16.

34. Shoji Y, Akhtar S, Periasamy A, Herman B, Juliano RL. Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. *Nucleic Acids Res.* 1991;19:5543-5550.

35. Akhtar S. Antisense technology: selection and delivery of optimally acting antisense oligonucleotides. *J Drug Target.* 1998;5:225-234.

36. Smith L, Andersen KB, Hovgaard L, Jaroszewski JW. Rational selection of antisense oligonucleotide sequences. *Eur J Pharm Sci.* 2000;11:191-198.

37. Patil SD, Rhodes DG. Influence of divalent cations on the conformation of phosphorothioate oligodeoxynucleotides: a circular dichroism study. *Nucleic Acids Res.* 2000;28:2439-2445.

38. Agrawal S, Tan W, Cai Q, Xie X, Zhang R. In vivo pharmacokinetics of phosphorothioate oligonucleotides containing contiguous guanosines. *Antisense Nucleic Acid Drug Dev.* 1997;7:245-249.

39. Akhtar S, Hughes MD, Khan A, et al. The delivery of antisense therapeutics. *Adv Drug Deliv Rev.* 2000;44:3-21.

40. Crooke ST. Vitravene another piece in the mosaic. *Antisense Nucleic Acid Drug Dev.* 1998;8:vii-v3.

41. Merdan T, Kopecek J, Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Adv Drug Deliv Rev.* 2002;54:715-758.

42. Aigner A, Juhl H, Malerczyk C, Tkybusch A, Benz CC, Czubayko F. Expression of a truncated 100 kDa HER2 splice variant acts as an endogenous inhibitor of tumor cell proliferation. *Oncogene.* 2001;20:2101-2111.

43. Zhang L, Gasper WJ, Stass SA, Ioffe OB, Davis MA, Mixson AJ. Angiogenic inhibition mediated by a DNAzyme that targets vascular endothelial growth factor receptor 2. *Cancer Res.* 2002;62:5463-5469.

44. Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin Chem.* 1999;45(9):1628-1650.

45. de Soultrait VR, Lozach P-Y, Altmeyer R, Tarrago-Litvak L, Litvak S, Andreola ML. DNA aptamers derived from HIV-1 RNase H inhibitors are strong anti-integrase agents. *J Mol Biol.* 2002;324:195-203.

46. Chaloin L, Lehmann MJ, Sczakiel G, Restle T. Endogenous expression of a high-affinity pseudoknot RNA aptamer suppresses replication of HIV-1. *Nucleic Acids Res.* 2002;30:4001-4008.

47. Bertrand J-R, Pottier M, Vekris A, Opolon P, Maksimenko A, Malvy C. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochem Biophys Res Commun.* 2002;296:1000-1004.

48. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet.* 2002;3:737-747.

49. Scherr M, Morgan MA, Eder M. Gene silencing mediated by small interfering RNAs in mammalian cells. *Curr Med Chem.* 2003;10:245-256.

50. Kurreck J. Antisense technologies: improvement through novel chemical modifications. *Eur J Biochem.* 2003;270:1628-1644.

51. Martinez MA, Gutierrez A, Armand-Ugon M, et al. Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. *AIDS.* 2002;16(18):2385-2390.

52. Zamore PD, Aronin N. siRNAs knock down hepatitis. *Nat Med.* 2003;9(3):266-267.

53. Ge Q, McManus MT, Nguyen T, et al. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc Natl Acad Sci U S A.* 2003;100:2718-2723.

54. Wong IHN. Methylation profiling of human cancers in blood: molecular monitoring and prognostication (review). *Int J Oncol.* 2001;19:1319-1324.

55. Bennett RM. As nature intended? The uptake of DNA and oligonucleotides by eukaryotic cells. *Antisense Res Dev.* 1993;3:235-241.

56. Le Doan T. Cell binding and internalisation of oligonucleotides. *S.T.P. Pharma Sciences.* 2001;11:75-82.

57. Crooke ST. Delivery of oligonucleotides and polynucleotides. *J Drug Target.* 1995;3:185-190.

58. Hughes MD, Hussain M, Nawaz Q, Sayyed P, Akhtar S. The cellular delivery of antisense oligonucleotides and ribozymes. *Drug Discov Today.* 2001;6:303-315.

59. Liu F, Huang L. Development of non-viral vectors for systemic gene delivery. *J Control Release.* 2002;78(1-3):259-266.

60. Akhtar S, Juliano RL. Cellular uptake and intracellular fate of antisense oligonucleotides. *Trends Cell Biol.* 1992;2:139-144.

61. Wu-Pong S. Alternative interpretations of the oligonucleotide transport literature: insights from nature. *Adv Drug Deliv Rev.* 2000;44:59-70.

62. Holmes AR, Dohrman AF, Ellison AR, Goncz KK, Gruenert DC. Intracellular compartmentalization of DNA fragments in cultured airway epithelial cells mediated by cationic lipids. *Pharm Res.* 1999;16:1020-1025.

63. Kamiya H, Tsuchiya H, Yamazaki J, Harashima H. Intracellular trafficking and transgene expression of viral and non-viral gene vectors. *Adv Drug Deliv Rev.* 2001;52:153-164.

64. Tachibana R, Harashima H, Shinohara Y, Kiwada H. Quantitative studies on the nuclear transport of plasmid DNA and gene expression employing nonviral vectors. *Adv Drug Deliv Rev.* 2001;52:219-226.

65. Brown MD, Schatzlein AG, Uchegbu IF. Gene delivery with synthetic (non viral) carriers. *Int J Pharm.* 2001;229(1-2):1-21.

66. Luo D, Saltzman WM. Synthetic DNA delivery systems. *Nat Biotechnol.* 2000;18:33-37.

67. Agrawal S, Zhang R. Pharmacokinetics of oligonucleotides. *Ciba Foundation Symposium.* 1997;209(Oligonucleotides as Therapeutic Agents):60-78.

68. Yu RZ, Geary RS, Leeds JM, et al. Pharmacokinetics and tissue disposition in monkeys of an antisense oligonucleotide inhibitor of ha-ras encapsulated in stealth liposomes. *Pharm Res.* 1999;16:1309-1315.

69. Tam P, Monck M, Lee D, et al. Stabilized plasmid-lipid particles for systemic gene therapy. *Gene Ther.* 2000;7:1867-1874.

70. Kunisawa J, Nakagawa S, Mayumi T. Pharmacotherapy by intracellular delivery of drugs using fusogenic liposomes: application to vaccine development. *Adv Drug Deliv Rev.* 2001;52:177-186.

71. Hope MJ, Mui B, Ansell S, Ahkong QF. Cationic lipids, phosphatidylethanolamine and the intracellular delivery of polymeric, nucleic acid-based drugs. *Mol Membr Biol.* 1998;15:1-14.

72. Jaaskelainen I, Moenkkoenen J, Urtti A. Oligonucleotide-cationic liposome interactions. A physicochemical study. *Biochim Biophys Acta.* 1994;1195:115-123.

73. Jaaskelainen I, Stemberg B, Monkkonen J, Urtti A. Physicochemical and morphological properties of complexes made of cationic liposomes and oligonucleotides. *Int J Pharm.* 1998;167(1-2):191-203.

74. Monnard P-A, Oberholzer T, Luisi P. Entrapment of nucleic acids in liposomes. *Biochim Biophys Acta.* 1997;1329:39-50.

75. McAllister DV, Allen MG, Prausnitz MR. Microfabricated microneedles for gene and drug delivery. *Annu Rev Biomed Eng.* 2000;2:289-313.

76. Regnier V, Tahiri A, Andre N, Lemaitre M, Le Doan T, Preat V. Electroporation-mediated delivery of 3'-protected phosphodiester oligodeoxynucleotides to the skin. *J Control Release.* 2000;67(2-3):337-346.

77. Huang L, Viroonchatapan E. Introduction. In: Huang L, Hung M-C, Wagner E, ed. *Nonviral Vectors for Gene Therapy.* San Diego, CA: Academic Press; 1999:3-22.

78. Mah C, Byrne BJ, Flotte TR. Virus-based gene delivery systems. *Clin Pharmacokinet.* 2002;41:901-911.

79. Lotze MT, Kost TA. Viruses as gene delivery vectors: application to gene function, target validation, and assay development. *Cancer Gene Ther.* 2002;9:692-699.

80. McTaggart S, Al-Rubeai M. Retroviral vectors for human gene delivery. *Biotechnol Adv.* 2002;20:1-31.

81. Galimi F, Verma IM. Opportunities for the use of lentiviral vectors in human gene therapy. *Curr Top Microbiol Immunol.* 2002;261:245-254.

82. Martin KR, Klein RL, Quigley HA. Gene delivery to the eye using adeno-associated viral vectors. *Methods.* 2002;28(2):267-275.

83. Lien Y-HH, Lai L-W. Gene therapy for renal disorders: what are the benefits for the elderly? *Drugs Aging.* 2002;19:553-560.

84. Chamberlain JS. Gene therapy of muscular dystrophy. *Hum Mol Genet.* 2002;11:2355-2362.

85. Wolf JK, Jenkins AD. Gene therapy for ovarian cancer (review). *Int J Oncol.* 2002;21:461-468.

86. Walther W, Stein U. Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs.* 2000;60:249-271.

87. Lever AML. Gene therapy in the fight against AIDS. *Expert Opinion on Therapeutic Patents.* 1996;6:161-167.

88. Zhao W, Kobayashi M, Hosokawa M, Seth P. Adenoviral vectors for cancer gene therapy. *Curr Genomics.* 2002;3:163-180.

89. Hale SJ, Green NK. Viral approaches to cancer gene therapy. *Expert Opinion on Therapeutic Patents.* 2002;12:369-378.

90. Garton KJ, Ferri N, Raines EW. Efficient expression of exogenous genes in primary vascular cells using IRES-based retroviral vectors. *Biotechniques.* 2002;32(4):830,832,834,836,838,840,842-843.

91. Heider H, Verca SB, Rusconi S, Asmis R. Comparison of lipid-mediated and adenoviral gene transfer in human monocyte-derived macrophages and COS-7 cells. *Biotechniques.* 2000;28(2):260-265, 268-270.

92. Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med.* 2001;7:33-40.

93. Favre D, Provost N, Blouin V, et al. Immediate and long-term safety of recombinant adeno-associated virus injection into the nonhuman primate muscle. *Mol Ther.* 2001;4:559-566.

94. Timme TL, Hall SJ, Barrios R, Woo SLC, Aguilar-Cordova E, Thompson TC. Local inflammatory response and vector spread after direct intraprostatic injection of a recombinant adenovirus containing the herpes simplex virus thymidine kinase gene and ganciclovir therapy in mice. *Cancer Gene Ther.* 1998;5:74-82.

95. Arruda VR, Fields PA, Milner R, et al. Lack of germline transmission of vector sequences following systemic administration of recombinant AAV-2 vector in males. *Mol Ther.* 2001;4:586-592.

96. Tenenbaum L, Lehtonen E, Monahan PE. Evaluation of risks related to the use of adeno-associated virus-based vectors. *Curr Gene Ther.* 2003;3:545-565.

97. Flotte TR, Laube BL. Gene therapy in cystic fibrosis. *Chest.* 2001;120(suppl 3):124S-131S.

98. Raper SE, Chirmule N, Lee FS, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab.* 2003;80(1-2):148-158.

99. Owens RA. Second generation adeno-associated virus type 2-based gene therapy systems with the potential for preferential integration into AAVS1. *Curr Gene Ther.* 2002;2:145-159.

100. Nyberg-Hoffman C, Aguilar-Cordova E. Instability of adenoviral vectors during transport and its implication for clinical studies. *Nat Med.* 1999;5:955-957.

101. Cao B, Mytinger JR, Huard J. Adenovirus mediated gene transfer to skeletal muscle. *Microsc Res Tech.* 2002;58:45-51.

102. Bachrach E, Pelegrin M, Piechaczyk M, Pedersen FS, Duch M. Efficient Gene Transfer into Spleen Cells of Newborn Mice by a Replication-Competent Retroviral Vector. *Virology.* 2002;293:328-334.

103. Fattal E, Delattre J, Dubernet C, Couvreur P. Liposomes for the delivery of nucleotides and oligonucleotides. *S.T.P. Pharma Sciences.* 1999;9:383-390.

104. Fattal E, Dubernet C, Couvreur P. Liposome-based formulations for the delivery of oligonucleotides. *S.T.P. Pharma Sciences.* 2001;11:31-44.

105. Pedroso de Lima MC, Simoes S, Pires P, Faneca H, Duzgunes N. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Adv Drug Deliv Rev.* 2001;47(2-3):277-294.

106. Hwang SJ, Davis ME. Cationic polymers for gene delivery: designs for overcoming barriers to systemic administration. *Curr Opin Mol Ther.* 2001;3:183-191.

107. Lemkine GF, Demeneix BA. Polyethylenimines for in vivo gene delivery. *Curr Opin Mol Ther.* 2001;3:178-182.

108. Lollo CP, Banaszczyk MG, Mullen PM, et al. Poly-L-lysine-based gene delivery systems: synthesis, purification, and application. *Methods Mol Med.* 2002;69:1-13.

109. Borchard G. Chitosans for gene delivery. *Adv Drug Deliv Rev.* 2001;52:145-150.

110. LeHoux JG, Grondin F. Some effects of chitosan on liver function in the rat. *Endocrinology.* 1993;132:1078-1084.

111. Florea BI, Meaney C, Junginger HE, Borchard G. Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and non-differentiated COS-1 cell cultures. *AAPS PharmSci.* 2002;4(3):E12.

112. Koping-Hoggard M, Tubulekas I, Guan H, et al. Chitosan as a non-viral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Ther.* 2001;8:1108-1121.

113. Godbey WT, Mikos AG. Recent progress in gene delivery using non-viral transfer complexes. *J Control Release.* 2001;72(1-3):115-125.

114. Petersen H, Fechner PM, Fischer D, Kissel T. Synthesis, characterization, and biocompatibility of polyethylenimine-graft-poly(ethylene glycol) block copolymers. *Macromolecules.* 2002;35:6867-6874.

115. Leclercq F, Dubertret C, Pitard B, Scherman D, Herscovici J. Synthesis of glycosylated polyethylenimine with reduced toxicity and high transfecting efficiency. *Bioorg Med Chem Lett.* 2000;10:1233-1235.

116. Akinc A, Langer R. Measuring the pH environment of DNA delivered using nonviral vectors: implications for lysosomal trafficking. *Biotechnol Bioeng.* 2002;78:503-508.

117. Mumper RJ, Klakamp SL. Polymeric gene delivery systems for in vivo gene therapy. *Drug Targeting and Delivery.* 1999;10(Advanced Gene Delivery):143-173.

118. Ward CM, Pechar M, Oupicky D, Ulbrich K, Seymour LW. Modification of pLL/DNA complexes with a multivalent hydrophilic polymer permits folate-mediated targeting in vitro and prolonged plasma circulation in vivo. *J Gene Med.* 2002;4:536-547.

119. Jeong JH, Park TG. Poly(l-lysine)-g-poly(d,l-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. *J Control Release.* 2002;82:159-166.

120. Wolfert MA, Seymour LW. Chloroquine and amphipathic peptide helixes show synergistic transfection in vitro. *Gene Ther.* 1998;5:409-414.

121. Singla AK, Chawla M. Chitosan: some pharmaceutical and biological aspects-an update. *J Pharm Pharmacol.* 2001;53:1047-1067.

122. Kawamata Y, Nagayama Y, Nakao K, et al. Receptor-independent augmentation of adenovirus-mediated gene transfer with chitosan in vitro. *Biomaterials.* 2002;23:4573-4579.

123. Cui Z, Mumper RJ. Chitosan-based nanoparticles for topical genetic immunization. *J Control Release.* 2001;75:409-419.

124. Roy K, Mao H-Q, Huang SK, Leong KW. Oral gene delivery with chitosan-DNA nanoparticles generates immunological protection in a murine model of peanut allergy. *Nat Med.* 1999;5:387-391.

125. Gitsov I, Lambrych KR. Dendrimers: synthesis and applications. *Microspheres, Microcapsules & Liposomes.* 2002;5(Dendrimers, Assemblies, Nanocomposites):31-68.

126. Eichman JD, Bielinska AU, Kukowska-Latallo JF, Baker JR, Jr. The use of PAMAM dendrimers in the efficient transfer of genetic material into cells. *Pharm Sci Technol Today.* 2000;3:232-245.

127. Zinselmeyer BH, Mackay SP, Schatzlein AG, Uchegbu IF. The lower-generation polypropylenimine dendrimers are effective gene-transfer agents. *Pharm Res.* 2002;19:960-967.

128. Yoo H, Juliano RL. Enhanced delivery of antisense oligonucleotides with fluorophore-conjugated PAMAM dendrimers. *Nucleic Acids Res.* 2000;28:4225-4231.

129. Tejada-Berges T, Granai CO, Gordinier M, Gajewski W. Caelyx/Doxil for the treatment of metastatic ovarian and breast cancer. *Expert Rev Anticancer Ther.* 2002;2:143-150.

130. Marshall J, Yew NS, Eastman SJ, Jiang C, Scheule RK, Cheng SH. Cationic lipid-mediated gene delivery to the airways. In: Wagner E, ed. *Nonviral Vectors for Gene Therapy.* San Diego, CA: Academic Press; 1999:39-68.

131. Felgner JH, Kumar R, Sridhar CN, et al. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J Biol Chem.* 1994;269:2550-2561.

132. Hofland HEJ, Shephard L, Sullivan SM. Formation of stable cationic lipid/DNA complexes for gene transfer. *Proc Natl Acad Sci U S A.* 1996;93:7305-7309.

133. Kang S-H, Zirbes EL, Kole R. Delivery of antisense oligonucleotides and plasmid DNA with various carrier agents. *Antisense Nucleic Acid Drug Dev.* 1999;9:497-505.

134. Lappalainen K, Jaaskelainen I, Syrjanen K, Urtti A, Syrjanen S. Comparison of cell proliferation and toxicity assays using two cationic liposomes. *Pharm Res.* 1994;11:1127-1131.

135. Patil SD, Rhodes DG, Burgess DJ. Anionic liposomal delivery system for DNA transfection. *The AAPS Journal.* 2004;6:E29.

136. Dokka S, Toledo D, Shi X, Castranova V, Rojanasakul Y. Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes. *Pharm Res.* 2000;17:521-525.

137. Filion MC, Phillips NC. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim Biophys Acta.* 1997;1329:345-356.

138. Freimark BD, Blezinger HP, Florack VJ, et al. Cationic lipids enhance cytokine and cell influx levels in the lung following administration of plasmid:cationic lipid complexes. *J Immunol.* 1998;160:4580-4586.

139. Lee H, Williams SKR, Allison SD, Anchordoquy TJ. Analysis of self-assembled cationic lipid-DNA gene carrier complexes using flow field-flow fractionation and light scattering. *Anal Chem.* 2001;73:837-843.

140. Audouy S, Molema G, De Leij L, Hoekstra D. Serum as a modulator of lipoplex-mediated gene transfection: dependence of amphiphile, cell type and complex stability. *J Gene Med.* 2000;2:465-476.

141. Liu F, Qi H, Huang L, Liu D. Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration. *Gene Ther.* 1997;4:517-523.

142. Wheeler CJ, Felgner PL, Tsai YJ, et al. A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. *Proc Natl Acad Sci U S A.* 1996;93:11454-11459.

143. Fillion P, Desjardins A, Sayasith K, Lagace J. Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides. *Biochim Biophys Acta.* 2001;1515:44-54.

144. Lakkaraju A, Dubinsky JM, Low WC, Rahman Y-E. Neurons are protected from excitotoxic death by p53 antisense oligonucleotides delivered in anionic liposomes. *J Biol Chem.* 2001;276:32000-32007.

145. Patil SD, Rhodes DG. Conformation of oligodeoxynucleotides associated with anionic liposomes. *Nucleic Acids Res.* 2000;28:4125-4129.

146. Patil SD, Rhodes DG, Burgess DJ. Biophysical characterization of anionic lipoplexes. *Biochim Biophys Acta.-Biomembranes.* 2005; In Press.

147. Lee RJ, Huang L. Lipidic vector systems for gene transfer. *Crit Rev Ther Drug Carrier Syst.* 1997;14:173-206.

148. Guo W, Gosselin MA, Lee RJ. Characterization of a novel diolein-based LPDII vector for gene delivery. *J Control Release.* 2002;83:121-132.

149. Perrie Y, Gregoriadis G. Liposome-entrapped plasmid DNA: characterization studies. *Biochim Biophys Acta.* 2000;1475:125-132.

150. Venugopalan P, Jain S, Sankar S, Singh P, Rawat A, Vyas SP. pH-Sensitive liposomes: mechanism of triggered release to drug and gene delivery prospects. *Pharmazie.* 2002;57:659-671.

151. Maclean AL, Symonds G, Ward R. Immunoliposomes as targeted delivery vehicles for cancer therapeutics (Review). *Int J Oncol.* 1997;11:325-332.

152. Legendre JY, Szoka FC Jr. Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. *Pharm Res.* 1992;9:1235-1242.

153. Reddy JA, Low PS. Enhanced folate receptor mediated gene therapy using a novel pH-sensitive lipid formulation. *J Control Release.* 2000;64(1-3):27-37.

154. Sviridov YV, Zhdanov RI, Podobed OV, Tsvetkova TA, Konstantinov I, Bogdanenko EV. The LacZ gene transfer into L929 cells and [14C]-DNA tissue distribution following intraperitoneal administration of new pH-sensitive lipoplexes in mice. *Cytobios.* 2001;106:(suppl 1),7-14.

155. Xu L, Huang C-C, Huang W, et al. Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes. *Mol Cancer Ther.* 2002;1:337-346.

156. Shi N, Zhang Y, Zhu C, Boado RJ, Pardridge WM. Brain-specific expression of an exogenous gene after IV administration. *Proc Natl Acad Sci U S A.* 2001;98:12754-12759.

157. Khaw BA, da Silva J, Vural I, Narula J, Torchilin VP. Intracytoplasmic gene delivery for in vitro transfection with cytoskeleton-specific immunoliposomes. *J Control Release.* 2001;75(1-2):199-210.

158. Krauss WC, Park JW, Kirpotin DB, Hong K, Benz CC. Emerging antibody-based HER2 (ErbB-2/neu) therapeutics. *Breast Disease.* 2000;11:113-124.

159. Mannisto M, Vanderkerken S, Toncheva V, et al. Structure-activity relationships of poly(l-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. *J Control Release.* 2002;83:169-182.